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Implication of agathic acid from Utah juniper bark as an abortifacient compound in cattle

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ABSTRACT: Freshly ground Utah juniper [*Juniperus osteosperma* (Torr.) Little] bark was given via gavage at a dosage of 2.3 kg per cow twice daily to three pregnant cows starting on day 255 of gestation. All three cows aborted the calves after 4, 5 and 6 days of treatment. A fourth cow was dosed with Utah juniper needles and this cow calved early on day 268 of gestation with complications consistent with pine needle abortion. Chemical analysis of *Juniperus osteosperma* bark identified the major diterpene acid as the labdane acid known as agathic acid. Agathic acid was measured in the bark at a concentration of 1.5% (dry weight basis). Analysis of sera samples obtained from treated cows found detectable quantities of agathic acid, dihydroagathic acid and tetrahydroagathic acid, which are known serum metabolites of the abortifacient compound isocupressic acid. Based on the high incidence of induced abortion and detection of known metabolites in affected animals, the labdane acid known as agathic acid is considered to be an abortifacient compound in late-term pregnant cattle. Published in 2009 by John Wiley & Sons, Ltd.

Keywords: agathic acid; cattle; abortion; poisonous plants

INTRODUCTION

Ponderosa pine (*Pinus ponderosa* Laws) needles from trees on open rangelands of the western states have a long history of causing abortions in cattle when ingested in the late second or third trimester of pregnancy (MacDonald, 1952; Stevenson and James, 1972; James *et al*., 1977; Panter *et al*., 1990). Several years ago a labdane resin acid in the pine needles and bark of ponderosa pine (*Pinus ponderosa*) called isocupressic acid (**1,** Fig. 1) was identified as the major abortifacient agent in ponderosa pine needles (Gardner *et al*., 1994). This compound was later found in Monterey cypress (*Cupressus macrocarpa*) and lodgepole pine (*Pinus contorta*) and helped to explain similar abortion problems occurring with cattle eating these plants (Parton *et al*., 1996; Gardner *et al*., 1998).

Isocupressic acid was found to be metabolized in both the rumen and the liver through oxidative and reductive processes and the major metabolites identified included imbricatoloic acid (**2**), agathic acid (**3**), dihydroagathic acid (**4**) and tetrahydroagathic acid (**5**) (Lin *et al*., 1998; Gardner *et al*., 1999). It is not known if these metabolites are biologically active as abortifacients in late-term pregnant cattle.

The abortifacient activity of isocupressic acid is highly species-specific and unfortunately the only reliable biological assay is the bovine model (Short *et al*., 1992; James *et al*., 1994), and thus obtaining sufficient quantities of compounds to be tested for activity is problematic. However, if compounds of interest can be found as a major component in available plant material, the feeding of this plant to late-term pregnant cows can provide a reasonable alternative to testing large quantities of purified compound, which can be costly and difficult to obtain. The bark of Utah juniper (*Juniperus osteosperma*) was found to contain agathic acid as the major diterpene acid and thus this material was considered to be a good candidate for determining the abortifacient activity of this compound. Therefore the objective of this work was to test the abortifacient activity of Utah juniper bark in the bovine model as an indirect measure of the abortifacient activity of agathic acid.

MATERIAL AND METHODS

Plant Material

The bark and needles of Utah juniper (*Juniperus osteosperma*) were collected 21 April 2005 from standing trees 5 miles west of Park Valley, Utah (N 41° 49′ 49″; W 113° 25′ 34″). Plant material was allowed to dry at ambient temperature and then stored in burlap bags until feeding. The plant material was ground to pass a 2 mm screen the day before treatments and stored at 4 °C thereafter.

Animals

Four healthy, experimentally naïve, mixed-breed beef cows (weighing between 465 and 650 kg) from a herd with no history of abortion or reproductive disease were time-bred and confirmed pregnant via palpation and ultrasonography. The cows were maintained on standard complete diets including alfalfa hay with grain and protein supplements in outdoor paddocks. The pregnancies were monitored and verified via palpation and ultrasonongraphy prior to experimental dosing. For reference, the full-term pregnancy periods were defined from non-treated animals within the laboratory herd ($n = 11$) used in prior studies

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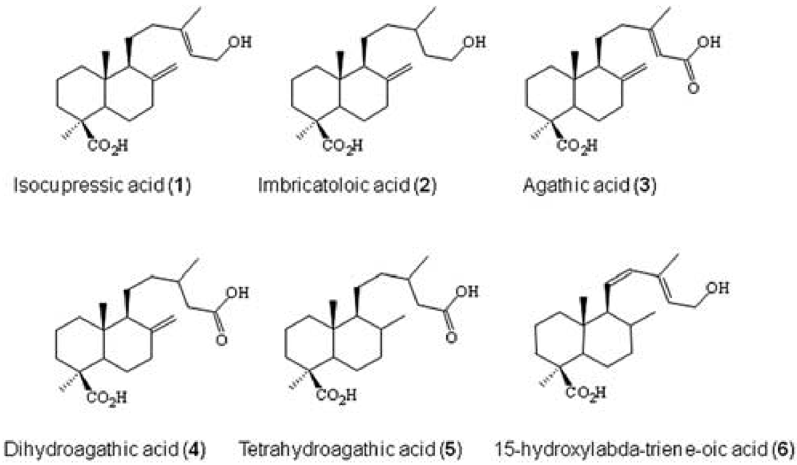

Figure 1. Chemical structures of labdane acids.

Table 1. Treated animals listed with the amount of plant material dosed daily, the corresponding dose of agathic acid and the number of days to parturition after start of treatment

Animal no.	Utah juniper	Daily plant dose (g kg^{-1})	Agathic acid daily dose (mg kg^{-1})	Days to parturition
7706	Bark	9.3	144	4
7700	Bark	8.8	134	5
7715	Bark	6.9	102	6
7713	Needles	9.8	20[a]	13 – dystocia
Control[b]	Alfalfa	–	–	29.4 ± 8.8

[a]For the needles the estimated dose was for the combined isocupressic acid and 15-hydroxylabda-trieneoic acid.
[b]Control animals are non-treated animals within the laboratory herd (n = 11) used in prior studies (Gardner *et al*., 1994, 1996). Average gestational period is 285 days or 30 days from start of treatment.

(Gardner *et al.*, 1994, 1996; Table 1). These control animals were treated identically to animals dosed with Utah juniper, including administration of ground alfalfa hay via stomach tube. For treated animals, three cows received Utah juniper bark and the last cow received Utah juniper needles. Ground plant material (2.3 kg) was administered in two doses per day (morning and afternoon) via stomach tube directly into the rumen starting on day 255 of pregnancy. This dose has been previously shown to be well tolerated and will consistently induce abortion with ponderosa pine needles (James *et al*. 1994). Dosing continued daily until abortion or day 265 (maximum of 20 doses). During treatment, the cattle were closely monitored for signs of parturition. Pine needle- and isocupressic acid-related abortions occur most frequently before day gestation day 270. Affected cattle often have difficult calving (dystocia), incomplete cervical dilation, weak uterine contractions and retained fetal membranes. Retained fetal membranes can result in endometritis and pyometria and should be closely monitored. In this study, if the membranes are not expelled after 24 h the animals were monitored, treated with uterine lavage and intrauterine antibiotic infusions. The combination of these clinical findings, early parturition, dystocia and retained placental membranes has been used to classify cattle in this model as a pine needle abortion (James *et al*., 1994). Although pine needle induced abortion can occur throughout pregnancy, the incidence is higher and more reproducible in late-term pregnancy. If the abortion is late in gestation, the calves are viable. However, they have immature lungs and are prone to increased respiratory problems and disease. These weak calves survive with appropriate, often extensive, nursing care. For serum agathic metabolite analysis, blood samples were collected via the jugular vein prior to the first dosage, 2 h post dosage and then daily until calving (James *et al*., 1994). All protocols for animal used in this research was done under veterinary supervision and reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Utah State University, Logan, Utah.

Analytical Methods

The concentration of agathic acid in the plant material was measured by gas chromatography after extraction using previously described methods (Gardner and James, 1999). Briefly, dry ground bark (0.100 g) was extracted in duplicate using the published procedure for isocupressic acid. To the dry extract was added 2.0 ml of methylene chloride. A 0.200 ml aliquot was transferred to a GC autosample vial and the solvent evaporated under flow of nitrogen. Pyridine (0.200 ml) containing 0.200 g ml^{-1} of heptadecanoic acid and 0.050 ml of BSTFA silyation reagent (Pierce Biotechnology, Rockford, IL. USA) were added and the sample capped and heated for 30 min at 60 °C. Before analysis 0.75 ml of methylene chloride was added. Calibration standards were prepared at 25, 50, 75 and 100 μg and were treated as above for silyation and dissolution. Gas chromatography was performed as previously described.

For analysis of sera samples for metabolites of isocupressic acid, a 1.0 ml aliquot of sera was placed into an 8 ml screw cap vial and 1.0 ml of saturated KH_2PO_4 was added. The samples were then extracted twice with chloroform (2.0 ml). After each extraction the samples were centrifuged to aid layer separation and the chloroform solution was withdrawn with a Pasteur pipette and passed through a filter of anhydrous sodium sulfate into a second 8 ml vial. The combined chloroform extracts were evaporated to dryness under a flow of nitrogen in a heat block at 60 °C. The samples were dissolved in 0.45 ml of pyridine and 0.050 ml of BSTFA silyation reagent and heated at 60 °C for 30 min. Samples were then analyzed for metabolites of isocupressic acid (agathic acid, dihydroagathic acid and tetrahydroagathic acid) by gas chromatography–mass spectrometry. Peak areas of the detected metabolites were measured from the total ion chromatogram and then plotted vs day collected to measure relative concentration of metabolites in the sera samples.

RESULTS

Chemical Analysis

Analysis of the Utah juniper bark by gas chromatography found the major labdane acid to be agathic acid (Fig. 2). The total available agathic acid concentration was measured at 1.52% (dry weight basis). The bark also contained other diterpene acids of pimaric- or abietane-type compounds but which are known not to be abortifacient (Stegelmeier *et al*., 1996). The needles of Utah juniper were found to contain low levels of isocupressic acid and the related 15-hydroxylabda-8(17),11,13-triene-19-oic acid (**6**) as the major components. Estimated concentrations were approximately 0.2% total labdane acids by dry weight in the juniper needles. This was less than 0.5% isocupressic acid that is considered to be the threshold concentration as it consistently produces abortion in cattle (Gardner and James, 1999).

In Vivo Bovine Assay

All three pregnant cows that were dosed Utah juniper bark aborted calves after 4–6 days of treatment (Table 1). All abortions were characterized by clinical signs typical of pine needle abortion including dystocia and retained placental membranes (James *et al*., 1994). The abortion induced in the single cow dosed with Utah juniper needles was delayed until gestation day 268; however, the parturition was clinically identical as it was complicated by weak uterine contractions and incomplete cervical dilation. More intense veterinarian assistance was required for this delivery. The fetal membranes were also retained.

A dose–response relationship between the amount of agathic acid and the number of days to parturition was also observed (Table 1). Daily doses of 144, 134 and 102 mg agathic acid kg^{-1} b.w. produced abortions at 4, 5 and 6 days after initiation of treatment respectively. The cow dosed with juniper needles received a much smaller dose (20 mg isocupressic acid and 15-hydroxylabda-trieneoic kg^{-1}) that took much longer, suggesting that this response was also related to dose.

Metabolite Assay

Agathic acid (**3**), dihydroagathic acid (**4**) and tetrahydroagathic aicd (**5**) were all detected in blood serum samples of cows dosed with bark or needles from Utah juniper. The relative amounts of the three metabolites measured from the sera samples are shown in Fig. 3A–C. In general, cows that aborted calves contained higher concentrations of metabolites. Significantly higher concentrations of agathic and dihydroagathic acid were found in the three cows dosed with Utah juniper bark vs the cow that was dosed with the needles. The animal dosed with needles of Utah juniper had the lowest serum concentrations of the labdane acids and aborted 13 days after initiation of treatment. The positive correlation between metabolite concentration in the serum was consistent for agathic acid and dihydroagathic acid but not for tetrahydroagathic acid (Fig. 3C).

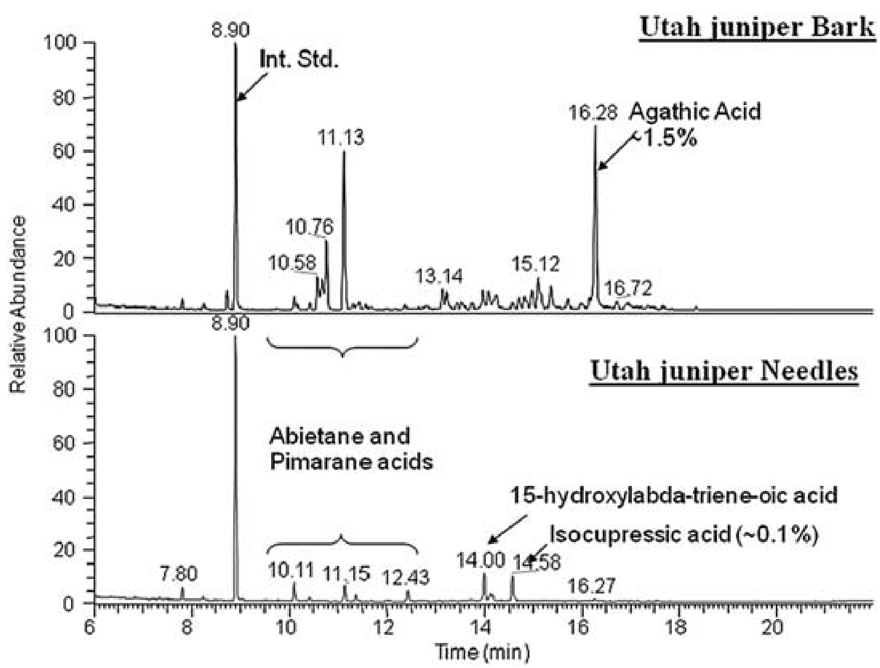

Figure 2. Chemical analysis of Utah juniper bark and needles. Gas chromatography–mass spectrometry chromatograms and the major diterpene acids identified from the plant material.

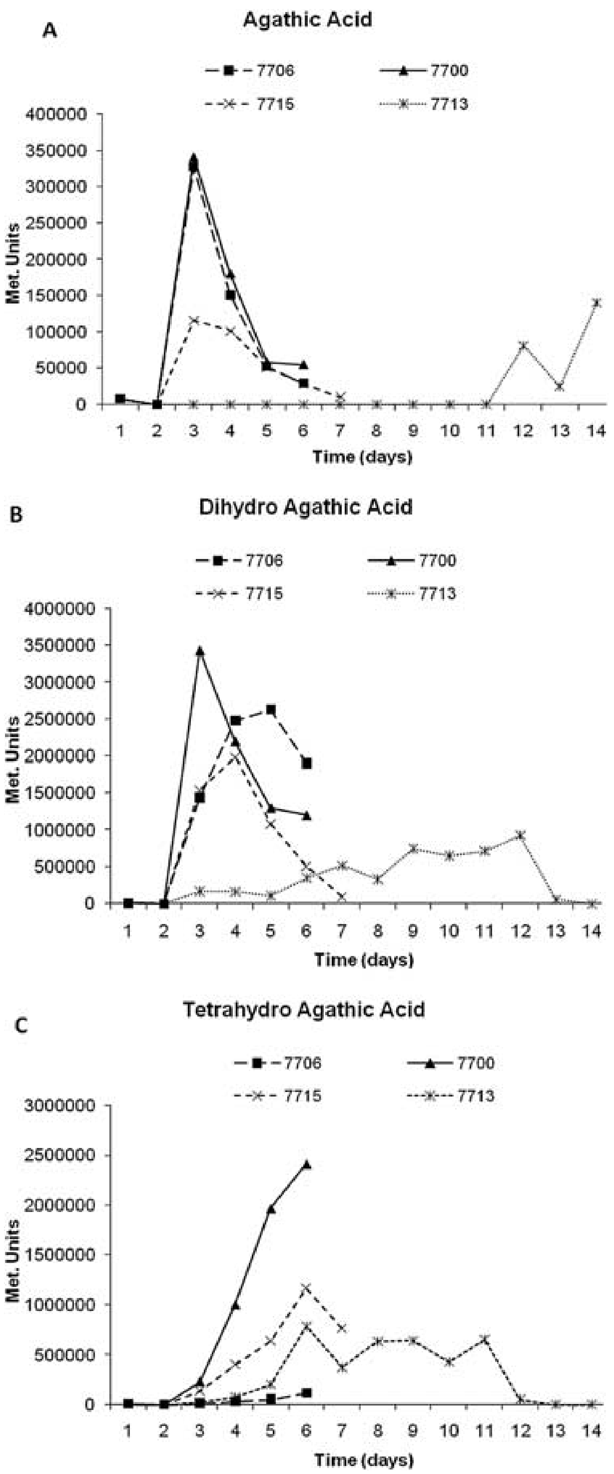

Figure 3. Relative concentration of labdane acids measured in sera of treated animals. Daily dose of agathic acid: cow 7706 (144 mg kg^{-1}), cow 7700 (134 mg kg^{-1}), cow 7715 (102 mg kg^{-1}) and cow 7713 (20 mg kg^{-1} labdane acids) (Table 1).

DISCUSSION

Based on chemical analysis of the plant material and results of the biological assay, we conclude that agathic acid in Utah juniper is abortifacient in cattle. Utah juniper bark is an excellent source of agathic acid with relatively little contamination by other labdane type diterepene acids. This information will be useful as further manipulation of agathic acid and the other isocupressic metabolites will be needed to ultimately identify the mechanism and cause of pine needle induced abortion.

The clinical results produced by agathic acid were essentially identical to those observed in dosing pregnant cows with isocupressic acid or plant material containing isocupressic acid (Gardner *et al*., 1994). In addition, the relative serum concentrations of agathic and dihydroagathic acid were as expected with the cow receiving the highest dose per b.w. showing the highest serum profile and also aborting first, followed by the cows dosed at 8.8 and 6.9 g kg^{-1} bw respectively. The nature of using a large animal assay does not facilitate, nor can we justify, the use of a large number of animals to completely define the dose–response relationship, but clearly the trend was observed, and when taken in context with similar experimental results with isocupressic acid (Gardner *et al*. 1994) the effect of dose on the rate of the induced abortion is evident.

The results of this study implicate agathic acid as an abortifacient compound in late-term pregnant cattle and adds to the list of known labdane type diterepene acids with abortifacient activity. The biochemical mechanism of the induced abortions is still unknown. Even so, agathic acid is clearly observed in the sera of animals that have aborted calves, whereas isocupressic acid is not observed in the sera, which suggests that agathic acid or dihydroagathic acid are better target molecules for use as a biomarker in diagnosis and investigating biochemical and physiological mechanisms of the pine needle-induced abortion.

Although Utah juniper poses little risk for the cattle industry and we have received no reports of cattle abortions caused by Utah juniper, it is an excellent source of agathic acid. The significance of the findings here are that we described the biological or toxicological activity of a particular compound, agathic acid, and this being the second labdane diterpene acid now to have such activity. It may be that the labdane acids in general have this unique toxicological effect in late-term pregnant cattle. This has important implications for other reported abortifacient plants such as broom snakeweeds, which have been reported to contain furano-labdane type acids (Roitman *et al*., 1994).

However, as closely related cedar and juniper species contain labdane resin acids and have been associated with abortion, livestock producers should be aware of this potential if conditions become such that cattle are forced to eat juniper or other related species. It is suggested that ranchers should use caution when placing pregnant cattle during late gestation in areas where trees or shrubs containing these types of compounds are available. Most importantly Utah juniper bark and agathic acid has research potential in further studies to understand the mechanism and possibly the solution to pine needle abortion in cattle.

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